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Testing the Feasibility of DNA Typing for Human Identification by PCR and an Oligonucleotide Ligation Assay

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Summary

The use of DNA typing in human genome analysis is increasing and finding widespread application in the area of forensic and paternity testing. In this report, we explore the feasibility of typing single nucleotide polymorphisms (SNPs) by using a semiautomated method for analyzing human DNA samples. In this approach, PCR is used to amplify segments of human DNA containing a common SNP. Allelic nucleotides in the amplified product are then typed by a colorimetric implementation of the oligonucleotide ligation assay (OLA). The results of the combined assay, PCR/OLA, are read directly by a spectrophotometer; the absorbances are compiled; and the genotypes are automatically determined. A panel of 20 markers has been developed for DNA typing and has been tested using a sample panel from the CEPH pedigrees (CEPH parents). The results of this typing, as well as the potential to apply this method to larger populations, are discussed.

Introduction

The analysis of DNA sequence polymorphisms has led to tremendous advances in the construction of genetic linkage maps and the mapping of human genetic diseases (Murray et al. 1994; Sheffield et al. 1995). It has also become an important tool in forensic and paternity testing (Sajantila et al. 1991; Hansen and Morling 1993; Pena and Chakraborty 1994).

Many types of DNA sequence polymorphisms are found in mammalian genomes (Yandell and Dryja 1989). Variations in the number of repeats or length of a DNA segment in a complex DNA sequence, such as VNTRs, or in simple tandem repeats (STRs), such as the di-, tri-, and tetranucleotide repeats, are the most commonly used polymorphisms for genetic and forensic analysis. Because the number of alleles at any given repeat locus tends to be large, the possibility that two different individuals might possess the same combination of alleles at even a few of these different loci is very small (Jeffreys et al. 1985*a*, 1985*b*, 1985*c*, 1986; Edwards et al. 1992; Lange 1993; Monson and Budowle 1993).

Estimation of fragment size by gel electrophoresis is the method of choice for typing VNTR or STR loci. Typing can be performed by RFLP analysis with a locus-specific probe (Botstein et al. 1980; Nakamura et al. 1988) or by amplifying across the repeated region with primers obtained from unique flanking sequences in the locus (Westwood and Werrett 1990; Reynolds et al. 1991; Edwards et al. 1992; Lee and Chang 1992; Roewer and Epplen 1992). Unlike disease diagnostics and genetic mapping, which require following the patterns of inheritance and segregation of polymorphisms in families, DNA typing in forensic situations requires determining whether the fragment sizes generated by two DNA samples are identical. Early applications of forensic DNA typing were criticized for a lack of experimental standards and quality control (Lander 1989). However, these issues have been addressed by the adoption of laboratory standards and the development of defined rules for declaring the odds of a match between samples when they are typed with specific markers (Lander and Budowle 1994; Cosso and Reynolds 1995).

Many of the current strategies for DNA typing rely heavily on electrophoretic analysis. With gel electrophoresis, polymorphic DNA fragments are discriminated solely on the basis of length; the use of length as a discriminating factor can be problematic for some markers because of gel-to-gel variations resulting from sequence-dependent mobility shifts (Gill et al. 1994; Holgersson et al. 1994; Jin and Chakraborty 1995). In addition, the throughput for gel-based typing approaches can be limiting, and the results for some markers are not easily interpreted in an automated fashion (Gill et al. 1994; Holgersson et al. 1994; Perlin et al. 1995). In order for DNA typing to become more automated, typing methods must become easier, faster, and amenable to simpler interpretation.

With a few exceptions (Walsh et al. 1991; Comey et

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al. 1993; Hochmeister et al. 1995), scientists have largely ignored another type of sequence polymorphism for DNA typing: single nucleotide polymorphisms (SNPs). These are usually diallelic and thus are inherently less informative than polymorphisms with multiple alleles; nonetheless, they offer several striking advantages for forensic testing: (1) SNPs are the most common and widely distributed type of DNA polymorphism in the human genome, with a frequency estimated at 1 in 800 nucleotides (Kwok et al. 1994); (2) SNPs are often more reliably amplified by PCR than repeat variations, which can lead to the formation of artifact bands (Kimpton et al. 1993; Murray et al. 1993; Whitaker et al. 1995); (3) since SNPs are diallelic, allele frequencies are easily determined and can be estimated in a population in a number of ways (Syvänen et al. 1992; Kwok et al. 1994); and (4) techniques for analyzing diallelic SNPs are also easier to automate on a large scale, and the results are easily interpreted by a computer (Nickerson et al. 1990; Nikiforov et al. 1994; Syvänen and Landegren 1994). With automation, large numbers of diallelic markers can be processed quickly to yield a highly informative system for forensic identification (Nickerson et al. 1990; Fodor et al. 1993; Nikiforov et al. 1994; Pease et al. 1994).

In the present study, we tested the feasibility of an SNP-based system for DNA identification. We utilized a strategy for DNA typing that combines DNA amplification via PCR with the specificity of the oligonucleotide ligation assay (OLA) (Landegren et al. 1988; Nickerson et al. 1990) to discriminate SNPs as well as unique insertions/deletions in a target sequence. OLA operates on the principle that DNA ligase can join two adjacent probes (~20 mers) only when they perfectly complement a denatured DNA target such as a PCR product. Even a single nucleotide mismatch at the junction of the probes will prevent ligation of the hybridized probes. By performing a separate ligation reaction for each of the allelic forms, the sequence of the DNA template at the polymorphic site can be identified on the basis of whether a positive or negative ligation event is observed for each reaction. The assay yields a simple numerical readout that can be interpreted directly by a computer: when the target DNA contains a base complementary to the probe, a colored product is formed; when it is not complementary, no color is formed. The entire assay is carried out in 96-well microtiter plates, and, since the assay requires only a single set of conditions, it has been semiautomated through the use of a robotic workstation for pipetting and plate-washing functions.

Material and Methods

Oligonucleotides

Oligonucleotide primers for both amplification and ligation reactions were synthesized using standard phosphoramidite chemistry on an Applied Biosystems 380A synthesizer. Ligation probes were modified either with a 5′ biotin group or with chemical phosphorylation using 5′ Phosphate-ON (Clontech). Biotinylated probes were purified using reverse-phase high-performance liquid chromatography on a Waters 715 UltraWISP. Phosphorylated probes were labeled with dUTP-digoxigenin by mixing 500 pmol of the oligonucleotide with 100 mM potassium cacodylate, 2 mM $CoCl_2$, 200 μM dithiothreitol, 2 μl of dUTP-digoxigenin (Boehringer Mannheim), and 2 μl of adenosine triphosphate (40 mM) with 24 U of terminal deoxynucleotidyl transferase and incubating at 37°C for 8 h.

DNA Amplification

PCR reactions were performed in a 96-well flexible plate on an Ericomp twin-plate thermocycler. The 20-μl reactions contained a buffer (10 mM Tris·HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$), the four deoxynucleotide triphosphates at 200 μM each, 1.25 mM amplification primers, 0.1% Triton H_2O, 0.5 U of *Taq* DNA polymerase and 20 ng of genomic DNA from each of the CEPH parents. The reactions were overlaid with mineral oil, and the DNA target was amplified by 40 cycles of 93°C for 30 s, 55°C for 45 s, and 72°C for 90 s.

Ligation Reactions

PCR amplification products were diluted with 45 μl of 0.1% Triton X-100 water. For each allele, a separate ligation reaction was assembled. Ligation probes (167 fmol each) in 10 μl of a solution containing 2 × ligase buffer (40 mM Tris·HCl (pH 8.0)/20 mM $MgCl_2$/2 mM dithiothreitol), 2 mM nicotinamide adenine dinucleotide, 25 mM KCl, and 0.167 U of Ampligase DNA Ligase (Epicentre) were mixed with 10 μl of the diluted amplified DNA samples in a V-bottomed 96-well polycarbonate microtiter plate. The reactions were overlaid with oil and placed in the thermocycler for 10 cycles of 93°C for 30 s and 58°C for 2 min. Reactions were stopped immediately after cycling with 10 μl of 0.1 M EDTA in 0.1% Triton H_2O. The reactions in their entirety were transferred to a 96-well flat-bottomed microtiter plate (Falcon) that had been coated with streptavidin (Sigma) (50 μl of 25 μg/ml incubated 1 h at 37°C) and blocked before use for 30 min at room temperature (RT) with 0.5% bovine serum albumin (Sigma) in 1 × PBS (ICN). Ligation products were allowed to capture at RT for 30 min, and the plate was then washed two times with NaOH wash (0.01 M NaOH/0.05% Tween 20) and 2 × with Tris wash (100 mM Tris·HCl [pH 7.5]/150 mM NaCl/0.05% Tween 20). Antidigoxigenin antibody (Boehringer Mannheim) was added to each well (40 μl of a 1:1,000 dilution in 1 × PBS). After 30 min incubation, the plate was washed six times with Tris wash. Substrate (25 μl of BRL ELISA amplification

Table 1

Diallelic SNP Markers Typed by PCR/OLA

Marker	Location	Variation	Allele Frequency[a]		Reference
			Allele 1	Allele 2	
AT3	1q23-q25.1	Length	.68	.32	Bock and Levitan (1983)
PROS1	3p11-q11	A→G	.59	.41	Syvänen et al. (1993)
BCHE	3q26.1-26.2	A→G	.73	.27	Bartels et al. (1990)
ARSB	5p11-q13	A→G	.61	.39	Syvänen et al. (1993)
CB26	7q35	T→G	.57	.43	Nickerson et al. (1992)
VB14	7q35	A→C	.38	.62	Present report
VB6.9	7q35	G→A	.63	.37	Hansen et al. (1992)
TYR	11q21	C→A	.35	.65	Giebel et al. (1990)
IGF2	11p15.5	A→G	.73	.27	Syvänen et al. (1993)
VWF	12p13.3-p13.2	A→G	.63	.37	Kunkel et al. (1990)
COL2A1	12q12-q13.2	T→C	.57	.43	Tsuneyoshi et al. (1991)
F7	13q34	C→T	.89	.11	Marchetti et al. (1993)
CA3	14q11.2	C→A	.68	.32	Nickerson et al. (1992)
HT3	14q11.2	C→T	.38	.62	Nickerson et al. (1992)
VA28	14q11.2	G→A	.53	.47	Present report
VA23	14q11.2	G→C	.47	.53	Present report
BCL2	18q21.33	A→G	.47	.53	Syvänen et al. (1993)
LDLR	19p13.2	T→C	.42	.58	Leitersdorf and Hobbs (1988)
PRNP	20pter-p12	A→G	.62	.38	Owen et al. (1990)

[a] Allele frequencies determined from PCR/OLA typing of 76 individuals from the CEPH pedigrees (CEPH parents).

was the case for the six markers typed together on one microtiter plate in this example (fig. 2). It is interesting to note that although only two sets of six markers were capable of uniquely identifying all individuals in the sample set, >5,000 combinations of 7 markers were capable of distinguishing all the members in this sample.

Discussion

The use of DNA fingerprinting is revolutionizing the field of forensic science. Coupled with PCR, DNA typing has the potential to exclude or include suspects based on trace amounts of blood or saliva recovered from the crime scene (Westwood and Werrett 1990; Hochmeister et al. 1991*a*, 1991*b*; Reynolds et al. 1991; Lee and Chang 1992; Roewer and Epplen 1992; Schneider and Rittner 1993). The uniqueness of individual DNA is accepted by the scientific and legal communities. While demands for DNA typing continue to grow, existing technologies are having difficulty keeping pace. Even in the technique's infancy, DNA typing for criminal trials and paternity disputes is backlogged many months. New applications for genotyping, such as military DNA profiles for the identification of remains or DNA profile databases of convicted felons, will tax the throughput of current gel-based systems even further (Hammond et al. 1994; McEwen and Reilly 1994). PCR promises to facilitate DNA typing both by providing a means to utilize DNA from alternative sources and by reducing the time required to prepare a DNA sample for analysis. Future development of DNA typing calls for a robust typing system that is compatible with PCR and allows rapid processing of samples, accurate determination of genotypes that can be subjected to quality-control measures, and efficient storage and exchange of data.

Many types of DNA polymorphisms can be applied to the analysis of DNA samples, including short tandem repeat polymorphisms (primarily tri- and tetranucleotide repeats, which are less prone to PCR slippage errors than dinucleotide repeats; Kimpton et al. 1993; Murray et al. 1993; Whitaker et al. 1995) or the most common type of sequence variation, SNPs. In the case of SNPs, there are several genotyping methods that can be combined with PCR to improve the throughput and interpretation of these diallelic markers, including genetic bit analysis (Nikiforov et al. 1994), allele-specific oligonucleotide hybridization using Taqman analysis (Livak et al. 1995), minisequencing (Syvänen et al. 1993), and OLA (Nickerson et al. 1990). One of the significant advantages of OLA is its ability to discriminate any nucleotide substitution or unique insertion/deletion by using a single set of assay conditions. Furthermore, it does not involve centrifugation or electrophoresis. The repetitive pipetting and washing steps required for the ELISA-based assay can be performed by a robotic workstation (Biomek 1000), thus allowing high sample throughput. Presently, 1,200 ligation assays/d can be processed by one technician and a single workstation (Nickerson et

Table 2

New PCR and OLA Probes Developed for DNA Typing

Marker	PCR Primer	Biotinylated Probe	Reporter Probe
AT3	AAGGTAGCAGCTTGTCCCTCTTTGC	1. B-AACAAACTTGGTTCATACCCA	pCCCTCTCTCATAGTTTTCTTTATG-D
	GTTCATACCCTCAAACTTGGTTAGG	2. B-TCTAGCCCTCTACCTGTAATT	
PROS1	GTACAGTTGGATCTGGATGAAGCC	1. B-TTAGAGCTCACTCATGTCCA	pTCAGTTTGGAAAAAGACAAA-D
	AGGTATTATAAGCAGAGAAAAGATGCC	2. B-TTAGAGCTCACTCATGTCCG	
BCHE	CTGAAACAAAAATGCCAGAAGG	1. B-AAACCCAAATGGCTAGAACA	pTGTTTAATTAAATTTCACAA-D
	AAAGAAAGAAATTGAACCAGGC	2. B-AAACCCAAATGGCTAGAACG	
ARSB	AGCCCCTTGCTGAAGCAGAAGG	1. B-TGACTGGCTGCCAACACTCG	pTGAAGCTGGCCAGGGGACAC-D
	CACGTCGAAGCCATCCAGAGGC	2. B-TGACTGGCTGCCAACACTCA	
VB14	ATGGGCCCCCAGCTCCTTG	1. B-TCCCAGAACACATAGGCAAA	pTTTTGGCCTTATTTTGTAGG-D
	AGGGGGAAATTCCTCTTCTCTTT	2. B-TCCCAGAACACATAGGCAAC	
VB6:9	GCGGAGCTCTGTCTCCTGGGA	1. B-CAGATACTGGAGTCTCCCAGG	pACCCCAGACACAA(G/C)ATCAC-D
	AACTGCATGCACAGAGATACAC	2. B-CAGATACTGGAGTCTCCCAGA	
TYR	GGATCAACACCCATGTTTAACGAC	1. B-GATGCACTGCTTGGGGGATA	pTGAAATCTGGAGAGACATTG-D
	CAACAAGAAGAGTCTATGCCAAGGC	2. B-GATGCACTGCTTGGGGGATC	
IGF2	CTTCTCCCTTGGACTTTGAG	1. B-CAGCAAAGAGAAAAGAAGGG	pCCCCAGAAATCACAGGTGGG-D
	GAGAAGGGAGATGGCGGTAC	2. B-CAGCAAAGAGAAAAGAAGGA	
VWF	GCAAAAGGAGCCTATCCTGTCG	1. B-GGCTGAAGGGCTCGAGTGTA	pCCAAAACGTGCCAGAACTAT-D
	CAGCCAGAGACACAGCCCATGC	2. B-GGCTGAAGGGCTCGAGTGTG	
COL2A1	TGGTGATGAAGGTTTCTGTTAGCCC	1. B-AAGACTCCTTTCCAAAGCTC	pCCTGCCTTTTAGTGACATCC-D
	TGTGGTCTCTCAGGGTGGAGGAGC	2. B-AAGACTCCTTTCCAAAGCTT	
F7	CTGATCTGTGTGAACGAGAA	1. B-AGCGCTCCTGTCGGTGCCAG	pGAGGGGTACTCTCTGCTGGC-D
	CAGGACACCCCGTCTGCCAG	2. B-AGCGCTCCTGTCGGTGCCAC	
VA28	ATGATGAAGTGTCCACAGGCT	1. B-TTGATCCTCAGAACACTAAG	pAGGGTAAAGTAGACTTCGTT-D
	GGTAGACGGCCGAGTCTCCGG	2. B-TTGATCCTCAGAACACTAAA	
VA23	GTCTAAGTGACAGAAGGAATG	1. B-GCAGCAAACAGGAGGTGACG	pCAGATTCCTGCAGCTCTGAG-D
	AATGTATAAAGTACTACGTCCTGA	2. B-GCAGCAAACAGGAGGTGACA	
BCL2	GTTGCTTTTCCTCTGGGAAGGATGG	1. B-TGGCGCACGCTGGGAGAACG	pGGGTACGATAACCGGGAGAT-D
	GCATCCCACTCGTAGCCCCTCTGCG	2. B-TGGCGCACGCTGGGAGAACA	
LDLR	CCGCCTCTACTGGGTTGACTCC	1. B-ATCTCAAGCATCGATGTCAAT	pGGGGGCAACCGGAAGACCAT-D
	TAAGCCACACCTCAAAGACGGC	2. B-ATCTCAAGCATCGATGTCAAC	
PRNP	GGTGGCTGGGGGCAGCCC	1. B-GGGGGGCCTTGGCGGCTACA	pTGCTGGGAAGTGCCATGAGC-D
	GTAACGGTCCTCATAGTCACTGCC	2. B-GGGGGGCCTTGGCGGCTACG	

al. 1990), and with the development of higher-density format microtiter plates and/or multiplex assay systems (V. Tobe, S. Taylor, and D. A. Nickerson, data not shown), the capacity could be increased to 5,000–10,000 assays/d. Since OLA yields accurate results with high signal-to-noise ratios using only 10% of the DNA generated by PCR amplification, there is sufficient sample remaining for duplicate testing or additional analysis by alternative methods for quality-control purposes. OLA offers the added advantage that it evaluates internal DNA sequences, so that the outcome of the assay is unaffected by the formation of nonspecific products during PCR amplification that can be a problem when less-than-optimal conditions or samples are used in these analyses (Sarkar et al. 1990; Hiltunen et al. 1994; Whitaker et al 1995). In addition, the assay readout can be directly transferred to a computer for data storage and analysis.

Table 3

Diallelic Markers and Theoretical Odds of a Match

	Allele Distribution[a]		
Odds of Match	10:90	30:70	50:50
1 in 10^6[b]	37[c]	16	14
1 in 10^7	43	19	16
1 in 10^8	49	22	19
1 in 10^9	56	24	21

[a] Frequency of marker alleles.

[b] Odds of a match were determined by $P = A^4 + 4A^2B^2 + B^4$, where A = frequency of allele A; and B = frequency of allele B.

[c] Marker requirements were determined by no. of markers = $\log x/\log p$, where x = discrimination level. Numbers rounded to the nearest whole number.

With automation, even large numbers of diallelic markers can be analyzed rapidly, allowing very high levels of discrimination. For instance, with the current marker panel, thirty individuals could be typed in a day with theoretical odds of a match in excess of 1 in 10^7 (Jeffreys et al. 1985*c*). However, theory and practice may not coincide, as shown in the present study: six markers were required to distinguish the 76 individuals in the test population, while, in theory, 5 markers should be sufficient for this task. Several plausible hypotheses

can be offered to account for this disparity: for example, it is a reflection of the small, relatively homogeneous population represented by the CEPH parent panel in which one sibling relationship and one set of grandparents exist; or, it reflects the occurrence of failed PCRs in the genotypings (<1%), which could influence marker requirements, since a genotype of 0 was considered a potential match. However, these findings further underscore the need for conservative calculations of the odds of match (Lander and Budowle 1994). In this regard, when large sets of diallelic markers (≥100) are available, alternative and highly conservative methods for calculating the odds of match can also be considered (Green 1992). For example, if a large panel of diallelic markers is used to type an individual's DNA, then a subset of markers for which the individual is known to be heterozygous can be identified. This marker subset can then be used to type the DNA sample(s) in question, and the odds of a match can be calculated by a simple formula, where the odds of match = $\frac{1}{2}^h$ (h is the number of heterozygous markers typed in the individual that match the DNA sample). This approach eliminates the need for a population database to determine the probability of a match between the individual and sample (since only markers known to be present and heterozygous in that individual and the sample are considered for calculating the odds of a match). It also decreases concerns surrounding DNA typing among different population groups, since only markers with known heterozygosity in the individual are used in calculating the odds of a match (Green 1992). We are currently in the process of developing a larger set of diallelic markers for DNA typing by this approach. However, it is clear from our initial study that the application of such new approaches to DNA typing as PCR/OLA will permit faster and simpler data interpretation and implementation of robust quality-control measures that can limit the errors and delays associated with large-scale DNA typing.

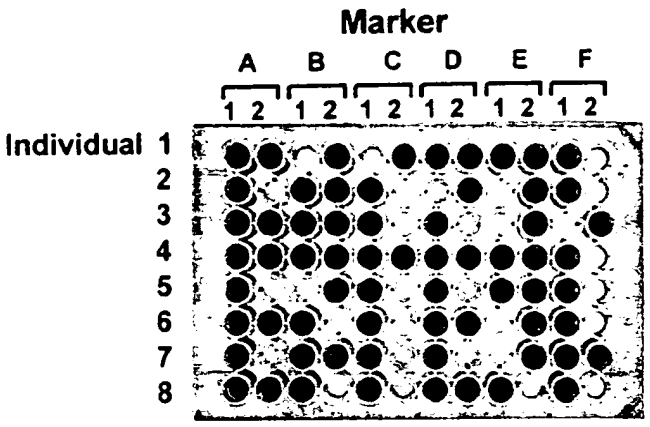

Figure 2 A representative OLA plate showing the genotyping of eight CEPH parent DNAs with six diallelic markers: marker A is PRNP, B is CA3, C is CB26, D is IGF2, E is LDLR, and F is COL2A1. The six different OLAs were performed in one microtiter plate by using a single set of assay conditions.

Table 4

DNA Typing by PCR/OLA of 76 Individuals with 20 Markers

20-Marker Genotype[a]	20-Marker Genotype[a]
1. 23201333312333133233	39. 32313323313313332133
2. 33331212333133113231	40. 13221113333113131231
3. 32323312112111313213	41. 13312311311112333213
4. 31313311323112233211	42. 23333333333313131211
5. 13231333213112333333	43. 31133111212103113313
6. 11212311113111311311	44. 33113112213212033331
7. 21331110221313333331	45. 12213131333312313233
8. 31233111333133332233	46. 12333232132111331331
9. 31312332333330313221	47. 11231122132310323333
10. 33333133323313133323	48. 31331133333112133313
11. 13113133213113332211	49. 22312323313312132211
12. 11131312132311111223	50. 13323112312112312233
13. 13222223133210331331	51. 31113233233131113311
14. 33133131113232121333	52. 12213332321232132333
15. 33332313322313333211	53. 11232323332313132331
16. 13213311213111133233	54. 23311113232213131333
17. 31332332233313133211	55. 13211213311312332121
18. 12331312133112113333	56. 12331132213212333231
19. 33113313313311131211	57. 31113332233310332213
20. 21322131332312133313	58. 31321132332133311311
21. 33232131312313233321	59. 13213232312333111313
22. 13233313211210121333	60. 12222111313111132331
23. 31232111332113313331	61. 33311330312132311333
24. 33111212333311221133	62. 22311111133112311331
25. 11331112113313312213	63. 31333333123112111233
26. 13322132233110112231	64. 11203321313333333211
27. 11132331323313123223	65. 32231133312313122333
28. 13331122332312113331	66. 32133112312311123331
29. 31213123313110231333	67. 33333313113112313213
30. 12113311212330111331	68. 31111133132212123311
31. 11221313212112123213	69. 23213332132332133333
32. 23111113313311131311	70. 13113310312311333121
33. 13332123313112312323	71. 32213332332332122333
34. 13231133213213133211	72. 13321113111313333131
35. 11233113332212113113	73. 33233332212332133333
36. 13311323333213113231	74. 11113133232130112111
37. 33323123312112233113	75. 12333113211113331113
38. 13233132312112312231	76. 32233130312332122331

[a] Each number represents a genotype (1 = homozygous allele 1; 2 = homozygous allele 2; and 3 = heterozygous alleles 1 and 2; 0 = failed PCR/OLA) for 20 markers, reported in the following order: CA3, CB26, LDLR, PRNP, COL2A1, IGF2, ARSB, BCL2, VA23, AT3, TYR, VWF, F7, HT3, BCHE, PROS1, VA28, VB14, VB6.9, SEX.

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